



Studies on the Development of Assay Methods for Phospholipase D Activity in vitro

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内容記述	この博士論文は内容の要約のみ公表しています
year	2014
その他のタイトル	ホスホリパーゼ D の活性測定法に関する研究
学位授与大学	筑波大学 (University of Tsukuba)
学位授与年度	2013
報告番号	12102甲第6941号
URL	http://hdl.handle.net/2241/00123743

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for Phospholipase D Activity *in vitro*
(Abstract)**

January 2014

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**A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
(Doctoral Program in Life Sciences and Bioengineering)**

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Introduction

Phosphatidylcholine (PC) is the most abundant phospholipid, representing 50% of the cellular membrane in eukaryotic cells. The cellular amount of PC, which is determined by a balance of synthesis and hydrolysis, plays important roles in cell proliferation, survival, apoptosis and homeostasis.

Phospholipase D (PLD), which is broadly distributed in bacteria, fungi, plants, and animals is known to hydrolyze the phosphodiester bond between the phosphate and choline of PC, thereby producing free choline and phosphatidic acid (PA). Importantly, the PLD-derived choline is an essential precursor of the neurotransmitter acetylcholine, while PA acts as a lipid second messenger that controls the actin cytoskeleton, vesicle trafficking for secretion and endocytosis, and receptor signaling. To understand the functions of PLD, it is important to determine its activity *in vitro*.

Current methods used to determine PLD activity include an enzyme-coupled spectrophotometric assay linking choline oxidation to the Amplex Red system, and a thin-layer chromatography (TLC) assay that monitors radiolabeled PC hydrolysis products. These methods are unique to PLD and can be used as specific assays for the enzyme. However, the Amplex Red reagent is unstable in the presence of thiols, and the radioactive PC label yields high non-specific background on the X-ray

film.

In recent years, matrix-assisted laser desorption/ionization-quadrupole ion trap-time-of-flight mass spectrometry (MALDI-QIT-TOF/MS) has been used as a novel technique for identifying low molecular weight compounds and biomolecules including peptides, proteins, and phospholipids. However, to date there has been no report measuring PLD-derived choline and PA using MALDI-QIT-TOF/MS. In this study, I have established a simple, sensitive, and rapid method for detecting choline and PA with MALDI-QIT-TOF/MS. By using 9-AA as a matrix, standard molecules of choline and PA are predominantly detected in positive and negative ion modes, respectively. Furthermore, I demonstrated that 9-AA can be used to detect PLD-derived choline and PA following the hydrolysis of PC. These data present a single-step method that allows for the evaluation of PLD activity by directly and simultaneously detecting choline and PA using MALDI-QIT-TOF/MS.

Results

Recent advances in MALDI-MS have allowed for the rapid and sensitive detection of phospholipids; however, the choice of an organic matrix is difficult because of requiring good absorptivity at the laser wavelength, good solubility in solvents, suitable acidity and basicity, and high ionization efficiency of molecules. First, to determine a proper matrix that enable to detect choline and PA, which are both products of the hydrolysis of PC by PLD, I tested the suitability of three matrices: DHBA, CHCA, and 9-AA. Accordingly, I prepared choline and PA as standards and performed MALDI-QIT-TOF/MS analysis using the three matrices. Interestingly, in both positive and negative ion modes, 9-AA provided clearly higher signal intensity and lower background as compared to DHBA and CHCA, suggesting that 9-AA was the most suitable matrix for detecting choline and PA. In addition, the standard calibration curves showed that choline and PA could be detected with linearity over the range from 0.05 pmol and 1 pmol, respectively.

I next investigated whether choline and PA yielded by hydrolysis of PC *in vitro* could be detected by MALDI-QIT-TOF/MS analysis using 9-AA matrix. A commercially available PLD from *Streptomyces sp.* and immunopurified mouse PLD2 proteins from HEK 293T cells were used as enzymes, followed by incubation with PC, and then the reaction solutions

were analyzed by MALDI-TOF/MS in both positive and negative ion modes.

The mass spectra of the reaction products showed molecular ions identical to the peaks for the standards of choline and PA only when incubated with the PLD. Furthermore, MS spectra of MS/MS analysis in negative ion mode showed the same mass-to-charge ratio peaks of PA standard. These results suggest that the utilization of the 9-AA matrix allows for the concomitant detection of choline and PA as the hydrolysis products of PC and this method can be used to evaluate PLD2 activity by directly detecting choline and PA as reaction products.

Discussion

Phospholipase D (PLD) enzymes hydrolyze the phosphodiester bond of phosphatidylcholine (PC), resulting in the production of free choline and phosphatidic acid (PA). PLD-derived products, especially PA, play important roles in regulating cell growth, survival, apoptosis, and homeostasis. Thus, measuring PLD activity contributes to the biochemical and molecular understanding of the biological functions of PLD.

Current progress matrices development us to analyze various cellular phospholipids using MALDI-TOF/MS. Here, I report a simple, sensitive, and rapid method for measuring PLD activity using MALDI-QIT-TOF/MS with 9-aminoacridine (9-AA) as matrix. The choice of matrix used for MALDI-TOF/MS depends on the mass range and chemical properties of the analytes. I presented an initial investigation using three different matrices (DHBA, CHCA, and 9-AA) for the detection of standard choline and PA at the same concentration. For each MALDI matrix, analysis type and sample type, the polarity (positive or negative) of the MALDI ion source must be determined experimentally in order to achieve the best results. Consider for instance that metabolite analysis with 9-AA achieves better results in negative ion mode, while protein analysis with DHBA performs better in positive ion mode. Previous studies have suggested that even when applied in higher concentrations (up to 3 mg/mL),

PA is not detectable in the negative ion mode, because PA bears two negative charges and the addition of one positive charge would be necessary in order to detect PA as a singly charged molecular ion. By contrast, when using 9-AA, this method could detect PA from 1 pmol and 9-AA dose give the best results for the PA in negative ion mode. Moreover, 9-AA also demonstrated low background and good sensitivity for selected PA fragment ions under collision-induced dissociation (CID) MS/MS. It is reasonable to suppose that as an acidic matrix, 9-AA transfers a proton to the PA molecule, forming a single negatively charged PA, $[M-H]^-$.

In conclusion, using the 9-AA-based MALDI-QIT-TOF/MS method, I can detect choline and PA, not only in standard molecules, but also in PLD-derived products from PC. This method will be useful for future studies on the biological functions of PLDs, and related questions of PC metabolism.